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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

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INVENTOR(S)		
Given Name (first and middle (if any))	Family Name or Surname	Residence (Street Address, city and either state or Foreign Country)
Per Olaf	Ekstrøm	

[ ] Additional inventors are being named on the separately numbered sheet(s) attached hereto

TITLE OF THE INVENTION (500 characters max)

Oscillating Temperature Capillary Electrophoresis and Uses Therefor

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[X] Specification *Number of Pages* [15]

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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

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[ ] Yes, the name of the U.S. Government agency and the Government contract number are: _____

Signature	Doreen M. Hogle	Date	November 12, 2003
Submitted by Typed or Printed Name	Doreen M. Hogle	Reg. Number	36,361

Rev. August 4, 1999

PATENT APPLICATION SERIAL NO. _____

U.S. DEPARTMENT OF COMMERCE
PATENT AND TRADEMARK OFFICE
FEE RECORD SHEET

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OSCILLATING TEMPERATURE CAPILLARY ELECTROPHORESIS AND USES THEREFOR

BACKGROUND OF THE INVENTION

Current efforts in discovery and screening of DNA variations (point mutations, single-nucleotide polymorphisms (SNPs) and other polymorphisms) are expected to yield valuable information on potential genetic risk factors as well as information on genetic variants that could lead to enhanced susceptibility for certain diseases. It is expected that discovery and massive screening of DNA polymorphisms will become essential for taylor-made drugs as well as disease gene association studies. Screening for and identification of SNPs and other polymorphisms will therefore require methods for separating variant DNA sequences from large samples.

Among the various experimental techniques for detection of DNA variations, DNA sequencing is the most universal approach. Although sequencing has been the "gold standard" for detecting polymorphisms for many years, a variety of alternative approaches for detection and screening have emerged, overcoming the high cost and occasional lack of sensitivity. These methods can rely on hybridization, allele-specific enzymatic reactions such as PCR, minisequencing, strand displacement, and/or cleavage, or on differences in physicochemical properties of the DNA variants such as the melting equilibrium, or affinity towards stationary phase. The results of these techniques can be read directly from the reaction mixture using plate readers or following separation of nucleic acids by capillary electrophoresis (CE), HPLC or mass spectrometry. The techniques employing CE, HPLC or mass spectrometry offer an additional means of identification of the variation from a characteristic pattern of

techniques, the sample throughput can be increased by processing multiple samples in parallel. This feature becomes useful when confronted with the vast amount of potential sequence targets to be scrutinized.

Electrophoresis has traditionally been used for separation of DNA fragments, DNA sequencing as well as general fragment analysis. A large family of slab-gel techniques dedicated to detection of DNA variations is based on differential melting of wild-type (taken to be a particular reference sequence) and mutant (a sequence that varies from the wild-type sequence) DNA fragments translated into an observable retention difference through electrophoretic sieving. One technique that utilizes this principle is denaturing gradient gel electrophoresis (DGGE), in which amplified fragments of wild-type and mutant sequences are resolved during their migration in a slab gel containing a gradient of chemical denaturant. DGGE is well established in clinical diagnostics due to its relative simplicity and ability to resolve close to 100% of mutations present in a sample for a given target sequence. Following DGGE, other variants of slab-gel mutant separation methods were developed including temporal temperature gradient gel electrophoresis (TTGE), in which the temperature is varied throughout separation and constant denaturing gel electrophoresis (CDGE), where the separation takes place at predetermined constant denaturing conditions.

In addition to the electrophoretic methods, an approach based on denaturing HPLC (dHPLC) has been used to identify polymorphsims. dHPLC uses an ion-pair chromatography separation principle combined with precise control of the column temperature and optimized mobile phase gradient for separation of mutant heteroduplexes. dHPLC can be easily automated and offers an option to collect the isolated heteroduplexes for further identifioation or confirmation by sequencing. However, the main potential of DGGE and dHPLC is mostly in discovery of novel mutations rather than screening due to their relatively low throughput of approximately 5 min per analysis.

The transition in DNA separations from traditional slab-gel electrophoresis to CE systems started in early 1990 and was later further accelerated by the Human

Genome Project. Separation of heteroduplexes was achieved at different temperatures controlled by means of Joule heating through adjustment of the separation voltage. In 1994, a method referred to as constant denaturing capillary electrophoresis (CDCE) was introduced as a capillary analog to CDGE, a constant denaturing electrophoresis performed on slab gels. For CDCE, the separation is carried out at an accurately maintained constant temperature at which the homo- and heteroduplex forms of wild-type and mutant sequences exhibit the best separation. An alternative approach was later used that overcomes the requirement for a very accurate temperature control by applying a simple temporal temperature gradient. Applying a temperature gradient, rather than a constant temperature, is useful especially in multi-capillary systems where maintaining accurate temperature across all capillaries is difficult. With a temperature gradient, each capillary reaches its temperature optimum, even if there is a difference in absolute temperature values among capillaries. The duration of such optimum separation conditions only depends on the overall slope of the temperature gradient.

The temperature gradient approach is very promising for detection of DNA variants, since it can be applied to many existing commercial multi-capillary CE systems with no additional requirements on instrument hardware. The typical run time is less than 60 minutes, depending on the resolution requirement (*i.e.*, gradient slope). With an automated 96-array capillary array instrument this results in a sample throughput of 1536 samples in 24 hours. It is clear that in order to apply this technology for massive screening of mutations or SNPs, the sample throughput needs to be further increased.

SUMMARY OF THE INVENTION

Described herein is a new type of DNA variation (polymorphism, mutation or SNP) screening technology based on capillary array electrophoresis. Sample DNA fragments of known sequence are PCR amplified and detected based on their differential migration in a polymer-filled capillary. A cycling (oscillating) temporal temperature gradient is used to compensate for local fluctuations in temperatures across

the multicapillary array. In addition, the application of short periodic temperature cycles allows a user to continue injecting subsequent sample plates between the temperature gradient cycles before the earlier samples appear in the detector. Using this novel approach, a dramatic and unexpected increase in separation throughput by more effective utilization of the separation capacity (volume) of each capillary is demonstrated.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A-1C are graphs showing temperature over time during different CE methods. FIG. 1A shows multiple-injection CE. FIG. 1B shows multiple- injection TGCE. FIG. 1C shows multiple-injection CGCE. The upper drawing illustrates the temperature profile. The simulated positions of separated sample zones are at the bottom.

FIGS. 2A and 2B. are spectral profiles showing a comparison of two temperature profile modes. FIG. 2A shows the separation of LTA variants in a single-sweep gradient mode. FIG. 2B shows the separation of LTA variants using a cycling temperature gradient mode. The peak resolution is fully maintained when a cycling gradient is applied.

FIGS. 3A and 3B show experimental measurements of temperature profiles inside MegaBACE 1000 capillary chamber. FIG. 3A depicts the typical profile of a single-sweep gradient mode. FIG. 3B shows a cycling temperature gradient mode. The instrument was equipped with high-temperature setting adaptor.

FIG. 4 is a fluorescence and temperature profile depicting the analysis of several samples for APC mutation detection using multiple-injections and fast cycling temperature gradient conditions. The three injections were performed every fourth temperature cycle. First injection is C homozygote, second injection is T homozygote and the last injection is C/T heterozygote.

FIG. 5 is an illustration of high throughput SNP scoring by CGCE in multicapillary format. Five different sample plates (FAM-labeled PCR fragments of

BRCA2) were consecutively injected during a cycling temperature gradient. Allele scoring was performed against a TMR-labeled internal mutant showing heterozygote profile with both allele peaks. The internal standard was also used as an "injection marker" for consecutive data processing by SNP Profiler software.

FIG. 6 is a schematic diagram depicting an overview of CGCE sample processing workflow.

DETAILED DESCRIPTION OF THE INVENTION

A description of preferred embodiments of the invention follows.

The present work extends technology for detection of DNA variants into a high throughput mode. Methods for screening multiple samples for the presence of somatic mutations after transferring a high-resolution technique of CDCE from single-capillary format to a commercial capillary array instrument had been described (Bjørheim, J. *et al.*, 2002, *Anal. Biochem.*, 304:200–205). Additional advantages of applying a temporal temperature gradient rather than maintaining an accurate constant temperature for increased reproducibility in a commercial multicapillary instrument were later described (Minarik, M. *et al.*, 2001, poster presented at ICHG Conference, Vienna, Austria). Described herein is an oscillating temperature gradient electrophoresis system useful for separating biomolecules, *e.g.*, DNA. The methods described herein can be used in combination with multiple-injection technology to dramatically increase the sample throughput (Mansfield, E. *et al.*, 2000, U.S. Patent No. 6,156,178).

In a multiple-injection experiment, samples are serially injected into the capillary (or array of capillaries) in periodical time intervals separated by short application of separation voltage. The main advantage of this approach is more efficient usage of the separation capacity (migration volume) of the capillary column. Following injection of the first sample (or a set of samples in capillary array), a so-called interval voltage is applied for a sufficient period of time (typically 2–5 min for short oligonucleotides) preventing overlap of the slowest peak from the sample with the fastest migrating peak from the next sample. After this period, the next sample is

injected resulting in a continuing process during which the first set of samples is reaching detectors, the following set is separating and new ones are being injected. In the most common version, the injections are repeated several times before the fastest migrating peaks from the first sample reach the detector. After the final injection, a run voltage is applied for a longer period of time (30–60 min) to drive all peaks from all injections pass the detection window. The maximum number of injections are determined experimentally. Indeed, the multiple-injection technique is applicable to samples containing compounds with relatively narrow windows to prevent overlap of bands from different injections (paper in preparation).

For separation of wild-type and mutant fragments based on differential melting, this condition applies. In most of these cases, the time difference between the fastest migrating peak from the mixture (usually the unincorporated primer) and the slowest migrating peak (usually the most denatured fragment) is typically around 10 min relative to the total analysis time of 30 - 40 min (Kristensen, A. *et al.*, 2001, *BioTechniques*, 33:650–654; Zhu, L. *et al.*, 2001, *Electrophoresis*, 22:3683–3687). The multiple-injection scheme could be directly applied to DNA separation, where samples would be serially injected and separated at a constant temperature (FIG. 1A).

A different situation occurs if a temperature gradient needs to be applied. In temperature gradient capillary electrophoresis (TGCE), the running temperature is gradually changed during the entire run. The fundamental assumption is that the samples have to be subjected to a proximity of optimum melting conditions over a sufficient duration of their migration in the capillary. With multiple injections, the samples eluting at the beginning would be subjected to different temperature ranges compared to the ones injected later as shown in FIG. 1B. In order to subject samples from all injections to identical melting conditions, the temperature has to be periodically changed to follow the profile of repeatedly injected samples. With this arrangement, each sample undergoes the same number of temperature gradient cycles and thus is subjected to the same melting conditions (FIG. 1C).

Others have subjected samples (PCR products) containing an artificial high-melting domain ("CG-clamp" or "GC-clamp") to a temperature gradient to cover a range of optimum melting conditions (Kristensen, A. *et al.*, 2001, *BioTechniques*, 33:650–654). A typical result of this single-sweep gradient experiment (TGCE) is shown in FIG. 2A. An LTA mutant was subjected to a descending temperature gradient starting at 52 °C and ending at 48 °C with a rate of 0.1 °C per min. During the gradient, the period of time at which the samples were subjected to their optimum melting conditions is given by the gradient slope and has direct impact on separation resolution (Minarik, M. et al., 2001, poster presented at ASHG Meeting, San Diego, CA). Described herein are methods for subsidizing this period with a series of cycles during which the separated samples are several times subjected to the melting optimum. FIG. 2B shows a separation of the same sample (LTA) subjected to a cycling temperature gradient. It can be seen that the resolution in this case is fully comparable to the single-sweep gradient experiment from FIG. 2A. It seems that the overall retardation effect due to partial denaturation in case of a cycling gradient is similar to a single-sweep gradient.

Most commercial capillary array instruments allow controlling temperature during the run. FIG. 3 shows the temperature profile of a single-sweep gradient (A) and atypical cycling gradient (B) recorded inside the capillary chamber of MegaBACE 1000 capillary array instrument equipped with high temperature setting. The unchanged separation performance of the cycling gradient compared to a single-sweep gradient allows for the application of the multiple-injection method. Depending on the actual application, the samples can be periodically injected either in every gradient cycle or once in every few cycles (e.g., every cycle, every 2 cycles, every 3 cycles, every 4 cycles, every 5 to 10 cycles, every 7 to 20 cycles, every 15 to 40 cycles, etc.). A single cycle can start at either high temperature limit or the low temperature limit. An example of multiple-injection cycling temperature gradient capillary electrophoresis (CGCE) with fast temperature cycling (5 minutes per cycle) is shown in FIG. 4. The APC mutant samples were injected in every fourth gradient cycle. During each cycle, a

temperature gradient going from 55 °C to 53 °C and back to 55 °C was applied. An internal standard containing 1:1 mixture of individually amplified wild-type and mutant fragments labeled with a TMR dye was included in each sample well. Since no heteroduplexes were formed following the PCR, the resulting separation patterns consist only of homoduplexes. It can be seen that a full separation of both wild-type and mutant homoduplexes of the internal standard was achieved within the individual interval windows. Clearly, a very high resolution of the peak separation is achieved under the cycling temperature gradient conditions, allowing identification of both alleles in a heterozygous sample by comparison to the peak pattern of the standard. Apart from just detecting a presence of DNA variation from the characteristic peak pattern in heterozygous samples, the complete separation of the two homoduplexes allows direct identification of homozygous genotypes. This is the key in automated SNP genotyping, where the two homozygous genotypes can be directly scored. An example of high-throughput analysis and automated scoring of SNPs is shown in FIG.. 5. A BRCA2 SNP was screened in various patients using the five injections with 45 °C - 43 °C cycling temperature gradient. Each sample included a 6 - carboxy - N,N,N',N' - tetramethylrhodamine (TAMRA)-labeled internal standard. The data was processed using SNP Profiler software which allows assignment of individual injection windows. The genotypes are directly determined from the positions of mutant homoduplex peaks co-eluting with the internal standard homoduplex peaks.

The total runtime of the experiment shown in FIG. 5 was less than 2 hours. It was estimated that, on a common 96-capillary instrument, five injections could feasibly be performed without any adverse effect on the separation matrix. The total runtime would then be 20 min of initial "dead" volume + 5 × 15 minutes separation window + 15 minutes final electrophoresis = 110 min. Considering 10 minute additional periods required for gel replacement in between runs, a total of 12 of similar multi-injection runs can be performed in less than 24 hours of operation. This represents an overall throughput of 5760 samples in 24 hours. Commercial genetic analyzers are usually equipped with four dye channels. Considering that up to three fluorescent dye channels

can be used to detect unknown samples (the last channel is assigned for the internal standard), it is clear that the potential capacity can be further increased 3-fold up to 17,200 samples in 24 hours on a single 96-capillary instrument. Unlike in other mutant or SNP scoring methods, the presented technology includes a very straightforward workflow shown in FIG. 6. Following the original PCR amplification, there is no sample cleanup required. Using a 96-format, PCR thermocycler enables complete automation from sample preparation to the multiple-injection CE analysis using robotic plate handlers.

EXEMPLIFICATION

Example 1.

In the present work, the general principle that applying a temporal temperature gradient in CE can further be extended into applying a periodical temperature cycle is demonstrated. PCR is employed with one of the primers extended by a high-melting domain (the "GC-clamp") to amplify a target DNA sequence surrounding the mutant or SNP marker. The PCR conditions are specific for each target sequence.

The application of periodic cycles allows a better compensation of the local temperature fluctuations inside the multicapillary oven. Rapid gradient cycling with rates of up to several cycles per minute showed better results in comparison with slower cycling intervals. In addition, the instrument hardware does not appear to be able to follow the rapid temperature changes inside the chamber resulting in a relative constant average temperature of the optical components. Periodic application of the temperature gradient enables usage of multiple injection technology, in which different samples are serially injected between the cycles and separated under the same revolving temperature conditions. Multiple-injections allow for significant increase in sample throughput. Considering the ease of sample preparation, PCR directly followed electrophoresis without any post-PCR treatment such as desalting or removal of unincorporated primers. Evaluation of the mutant presence or SNP genotype is performed solely based on an internal standard running in a separate spectral channel. In situations where a

slower migrating PCR fragment would directly overlap with a faster migrating primer peak from a subsequent injection, the spacing between the injections was adjusted. For a given mutant/SNP marker, the peak distance is very reproducible, therefore the injection spacing can be optimized accordingly. This technique represents a cost effective, simple and powerful tool for high-throughput scoring of DNA mutants and SNPs.

Chemicals

All experiments were performed using standard Mega-BACE. buffers and MegaBACE. LPA long-read matrix (Amersham Biosciences, Piscataway, NJ, USA). PCR primers were obtained from MedProbe (Oslo, Norway). The primer with GC clamp were labeled with 6-carboxyfluorescein (6-FAM) on the tested samples and tetramethylrhodamine (TMR) on the internal standards. Primer used for SNP in the lymphotoxin alpha (LTA) gene (accession number 153440, locus 6p21.3, NCBI reference SNP ID: rs 909253), 5' CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GCT GGT GGG TTT GGT TTT GG 3' (SEQ ID NO:1)and 5' GAG CAG AGG GAG ACA GAG AGA G 3' (SEQ ID NO:2). Primer used for mutation in Adenomatous polyposis coli (APC) gene (accession number 175100, locus 5q21-q22, 1p34.3-1p32.1, exon 15, codon 1450), 5'- CGG GCG GGG GCG GCG GGA CGG GCG CGG GGC GCG GCG GGC GAG CAT TTA CTG CAG CTT GCT 3' (SEQ ID NO:3)and 5' ACC TCC TCA AAC AGC TCA AA 3' (SEQ ID NO:4). Primer used for SNP in the breast cancer-2 (BRCA2) gene (accession number 600185, locus 13q12.3, NCBI reference SNP ID: rs573014), 5' CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GAA GG TAT GTG CAT TGT TTT T 3' (SEQ ID NO:5) and 5' CCG CAATAA AGC AAATAT TAC 3' (SEQ ID NO:6).

CE

All CE experiments were performed on an MegaBACE 1000 96-capillary DNA analysis system (Amersham Biosciences). The instrument was equipped with an

automated loading robot Caddy 1000 (Watrex Praha, Prague, Czech Republic), to allow for unattended automated operation. To reach high temperatures needed for some mutant separation, the temperature sensor was equipped with an additional resistor resulting in a positive offset of temperatures by approximately 10 °C. The temperature was recorded using Fluke logging thermometer with FlukeView software (Michell Instrument, San Marcos, CA, USA). The temperature profiles were constructed using MegaBACE configuration selector (MBCS) software (Genomac International, Prague, Czech Republic). The data was processed by SNP Profiler. software (Amersham Biosciences).

PCR reaction

Full blood from blood donors at Ulleval Hospital (Oslo, Norway) was anonymously collected and genomic DNA was extracted with QIAamp DNA Blood Mini Kit from Qiagen (Valencia, CA, USA). All reactions were performed on a PTC-200 thermocycler (MJ Research, Waltham, MA, USA), by mixing 50 ng of genomic DNA with 25 mM of each dNTP (Abgene, Epsom, UK), 10 *Taq* buffer, 1 unit of *Taq* polymerase (Applied Biosystems, Foster City, CA, USA) and 5 pmol of each primer (MedProbe) in a final volume of 25 L. Same cycling conditions were applied for amplification of all fragments. The cycling program included denaturation for 60 s at 94 °C, annealing for 60 s at 53 °C and elongation 60 s at 72 °C, for 35 cycles. Where applicable, heteroduplexes were formed by heating the PCR products at 94 °C for 5 min, then annealing the fragments at 65 °C for 60 min followed by slow cooling to 4 °C.

Optimization of cycling temperature range

For each target sequence, extended by the GC clamp, the theoretical melting temperature profile was first calculated using WinMelt. simulation program (Medprobe) based on Poland's algorithm [26]. A melt profile will show regions of theoretical high and low melting domains of a known sequence. Location of primers and GC clamps can be optimized by analyzing their effect on the overall fragment melting profile. The

temperature oscillation was within ± 1 ° C range from the melting temperature of the low-melting domain (target sequence).

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.

CLAIMS

What is claimed is:

1. A method for separating nucleic acids comprising electrophoresing a sample applied to a capillary gel electrophoresis matrix, wherein during electrophoresis, the temperature of the matrix is cycled at least two times, wherein one cycle is from a high temperature to a low temperature or from a low temperature to a high temperature.

2. The method of Claim 1, wherein the temperature is initially at a high temperature and the first cycle is from a high temperature to a low temperature.

3. The method of Claim 1, wherein the high temperature and/or low temperature is different for successive cycles.

4. The method of Claim 1, wherein the temperature is cycled from about 2 to 40 times.

5. The method of Claim 1, wherein the temperature is cycled about 20 times.

6. The method of Claim 1, wherein the high temperature is about 3 °C higher than the low temperature.

7. The method of Claim 1, wherein the temperature is between about 2 °C and about 15 °C higher than the lower temperature.

8. The method of Claim 1, wherein the higher temperature is between about 3 °C and about 10 °C higher than the lower temperature.

9. A method for estimating allele frequency comprising:

electrophoresing a sample applied to a capillary gel electrophoresis matrix, wherein during electrophoresis, the temperature of the matrix is cycled at least two times, wherein one cycle is from a high temperature to a low temperature or from a low temperature to a high temperature, thereby separating DNA molecules in the sample; and

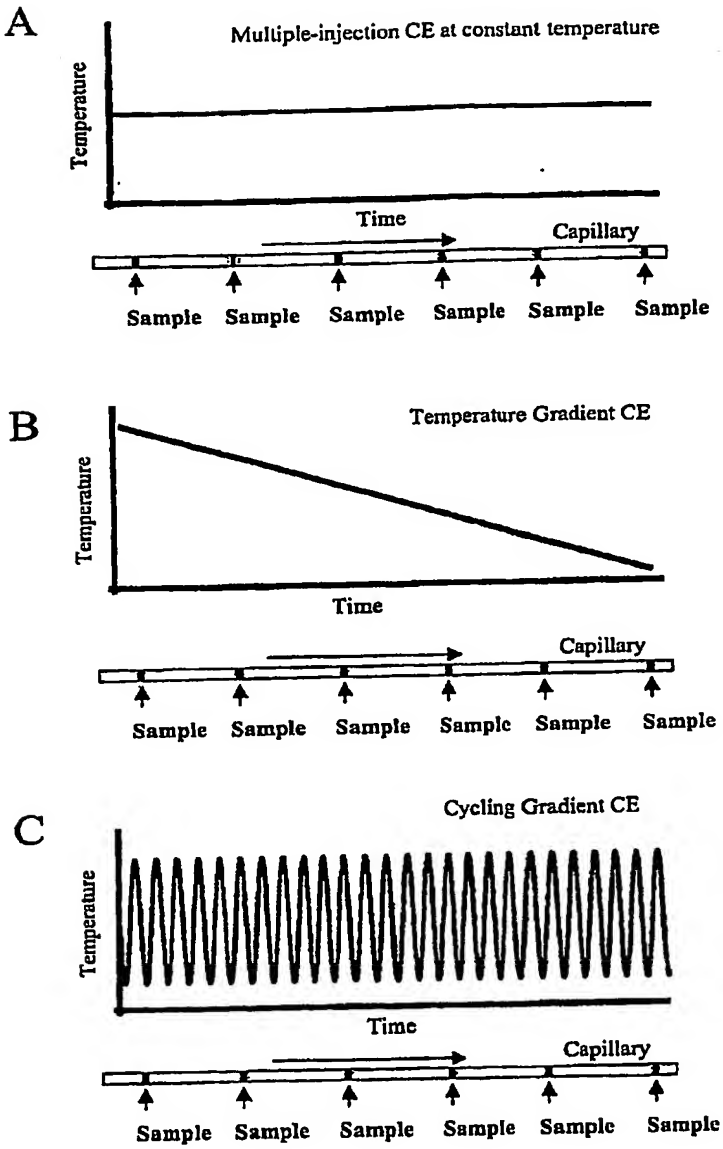
quantifying the variant sequences of the separated DNA molecules

thereby providing an estimate of the allele frequency for each variant DNA molecule.

OSCILLATING TEMPERATURE CAPILLARY ELECTROPHORESIS AND USES THEREFOR

ABSTRACT OF THE DISCLOSURE

Methods for separating biomolecules using oscillating temperature electrophoresis are disclosed. Uses for such methods are also disclosed including high throughput screening, estimation of allele frequencies, identification of polymorphisms, and separation of DNA molecules.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Time
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FIG. 1

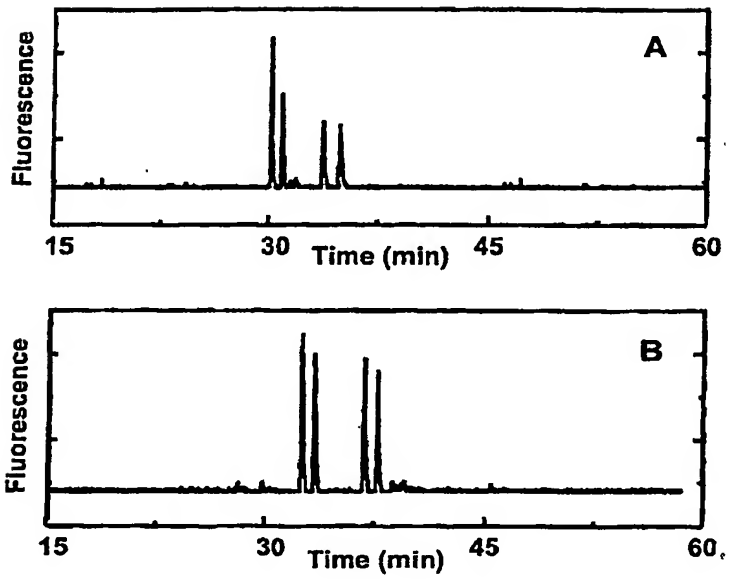
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FIG. 2

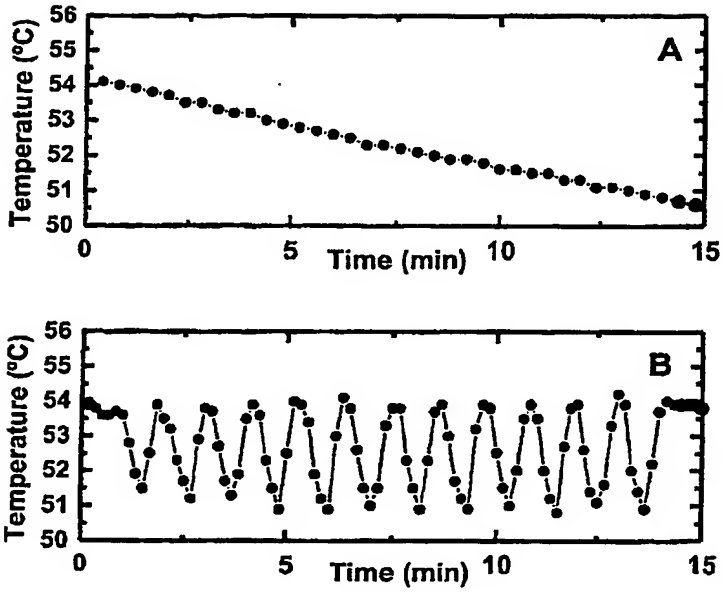
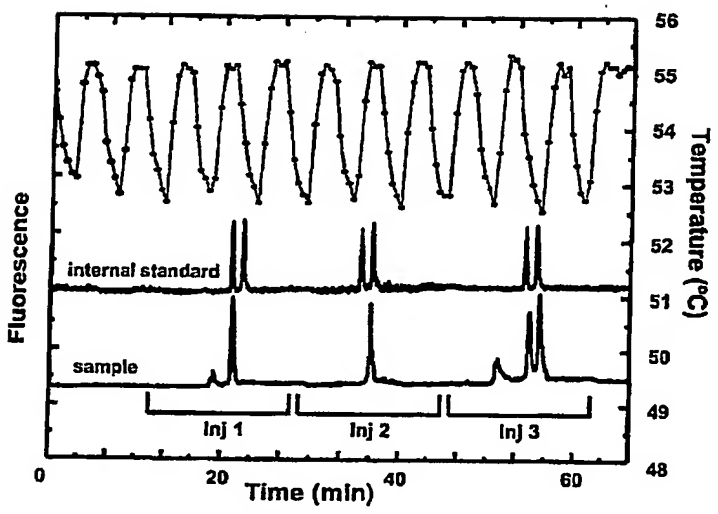


FIG. 3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48
Temperature (°C)

FIG. 4

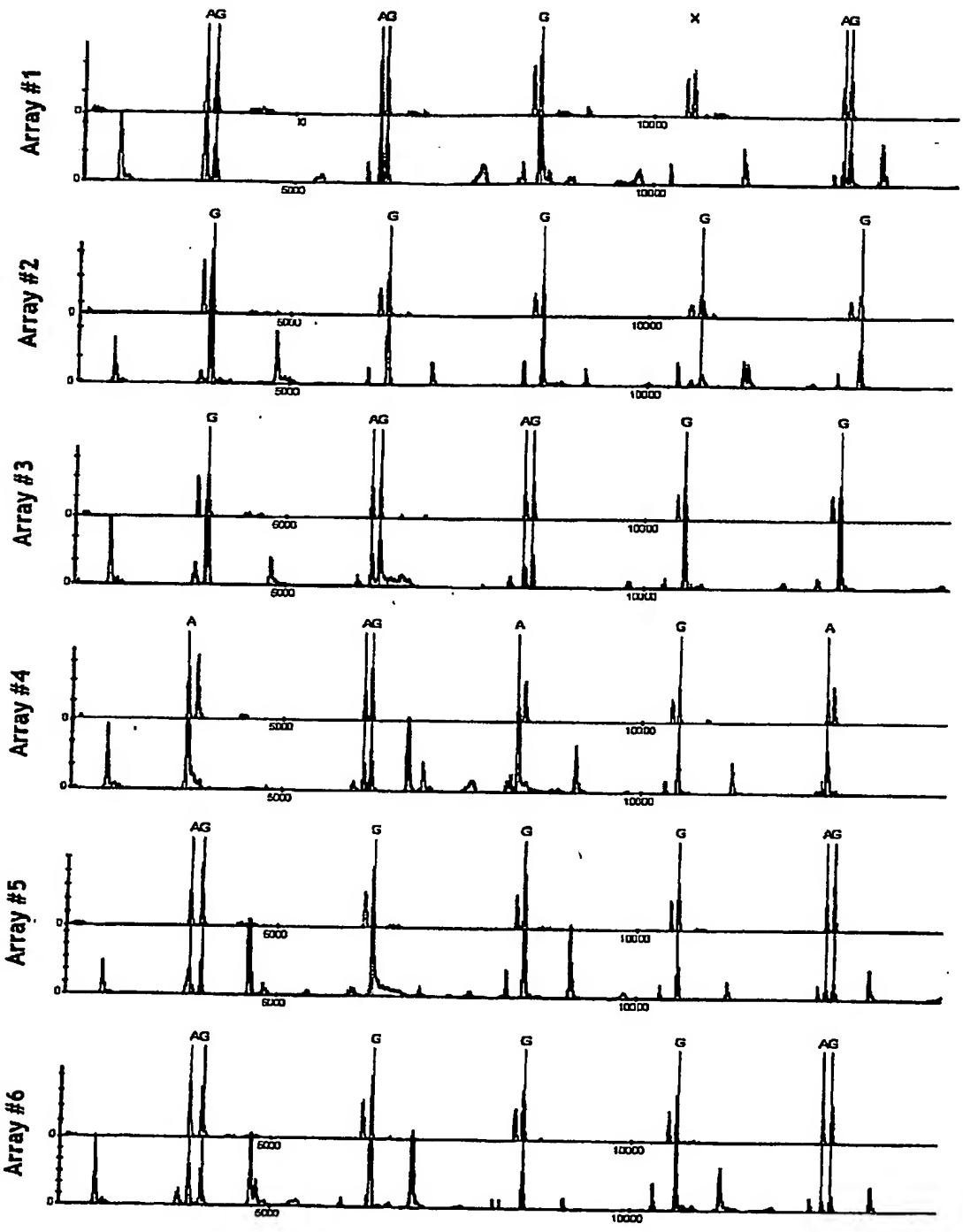
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FIG. 5

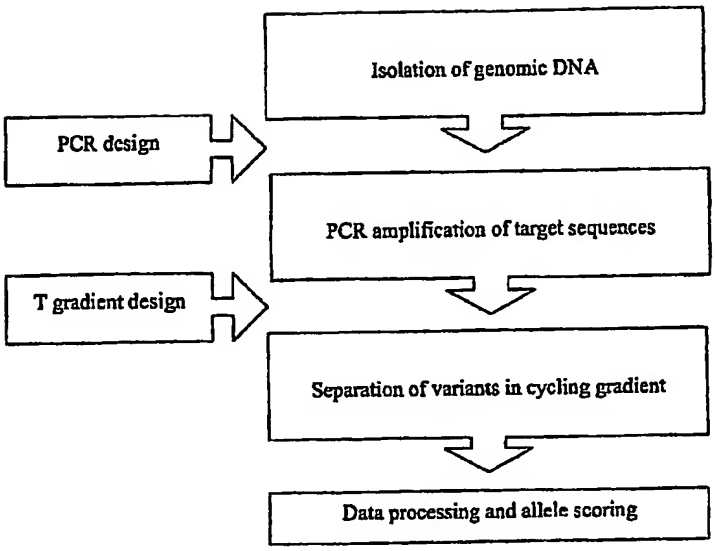
Isolation of genomic DNA
PCR design
PCR amplification of target sequences
T gradient design
Separation of variants in cycling gradient
Data processing and allele scoring

FIG. 6